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Identification and Characterization of the Expression Profile of Oligodendrocyte-Derived and Associated Proteins via Unilateral X-Irradation of the Rat Optic Nerve

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Identification and Characterization of the Expression Profile of Oligodendrocyte-Derived and Associated Protein Via Unilateral X-irradiation of the Rat Optic Nerve

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy at Virginia Commonwealth University

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Abstract

IDENTIFICATION AND CHARACTERIZATION OF THE EXPRESSION PROFILE OF OLIGODENDROCYTE-DERIVED AND ASSOCIATED PROTEINS VIA UNILATERAL X-IRRADATION OF THE RAT OPTIC NERVE

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Raymond J. Colello, D.Phil., Associate Professor, Department of Anatomy and Neurobiology

Recent studies examining cell-cell interactions during CNS development and following disease or trauma have highlighted our limited understanding of the in vivo functions of the myelinating cell of the CNS, the oligodendrocyte. With this in mind, our laboratory has developed techniques by which a profile of proteins derived from or regulated by oligodendrocytes can be elucidated. Specifically, we have demonstrated that oligodendrocytes can be selectively eliminated from one optic nerve of a rat by treating the animal with a unilateral exposure of X-irradiation at the time of birth. Consequently, this approach allowed us to experimentally create, within the same animal, one optic nerve devoid of oligodendrocytes and their progenitors (the X-irradiated side) and one optic nerve containing the normal oligodendrocyte population (the untreated side). Using this experimental animal model we hypothesized that uncharacterized proteins, derived from and regulated by oligodendrocytes, which are

crucial for CNS development can be identified. Specifically, by comparing protein profiles found within the normal myelinating optic nerve versus the X-irradiated optic nerve, where oligodendrocytes are absent, potential oligodendrocyte-derived proteins can be quickly identified. Further verification that these proteins are indeed related to oligodendrocytes and/or the processes of myelination can be obtained by their reappearance in the 2-D gel protein profile of P28 X-irradiated nerves that, as we have shown previously, undergo a delayed myelination. We then employed mass spectrometric analysis to determine the identities of oligodendrocyte derived/regulated proteins. In this thesis, I will begin by describing our current knowledge of the proteins expressed by oligodendrocytes and their role(s) in oligodendrocyte function. This will be followed by a detailed description of the experimental model system we utilized in an attempt to elucidate the complete repertoire of oligodendrocyte-regulated proteins. We will then describe the results generated from our methodology and discuss the implications of our findings in relation to the functional cooperation between oligodendrocytes and other cells of the developing central nervous system. The results generated from this project should lead to a clearer understanding of the role of oligodendrocytes and of the array of proteins whose expression patterns are associated with these cells during CNS development.

Chapter One

Introduction to CNS Myelin Biology

Oligodendrocytes, astrocytes and microglia, commonly known as *glial cells*, constitute the majority of the cell population in the central nervous system (CNS). The myelinating glial cell of the CNS, the oligodendrocyte, is essential for proper neuronal development, survival, and functioning in the mature CNS. The oligodendrocyte is morphologically distinguishable from other glial cells. It is small in size, has a dense cytoplasm and chromatic nucleus, is devoid of intermediate filaments and glycogen in the cytoplasm, and possesses a large number of microtubules in its extending processes, which contributes to its stability (Peters et al., 1991; Pfeiffer et al., 1993; Baumann and Pham-Dinh, 2001). Extending from the cell body of the oligodendrocyte is a variable number of processes, which contact and repeatedly envelop 1-2 mm segments of up to 50 neighboring axons, thus forming myelin sheaths. The persistent interactions between oligodendrocytes, other glial cell types, and neurons contribute to a cooperation that is necessary for proper CNS functioning (Baumann and Pham-Dinh, 2001).

Oligodendrogenesis

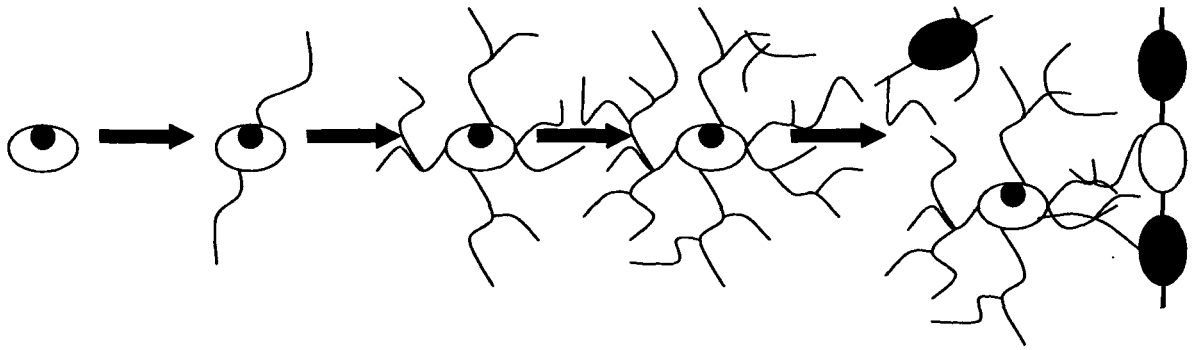
Prior to maturation and myelin formation, oligodendrocytes undergo many distinct phenotypic stages of development. Though extensive research has been done in an attempt to determine whether oligodendrocytes, astrocytes, and neurons are derivatives of a common precursor cell (Colello & Pott, 1997; Baumann & Pham-Dinh, 2001), much work remains to find the most accurate and plausible explanation. Irrespective of their

origin, subsequent to differentiation, oligodendrocyte precursor cells undergo a series of phenotypic stages characterized by the expression of antigenic markers, a change in their mitotic and migratory status, and alterations in morphological features **(Fig. 1.1)**.

The characterization of a number of antigenic cell surface markers both *in vivo* and *in vitro* has increased our knowledge about oligodendrocyte maturation. Throughout the maturational stages of an oligodendrocyte, a number of developmental markers are sequentially expressed, as others are lost (Pfeiffer et al., 1993) **(Fig. 1.1)**. The continuous expression and loss of stage-specific cell surface markers has allowed us to follow the progression of oligodendrocyte differentiation and maturation and has provided insight into specific functions carried out by the cell.

Oligodendrocyte precursor cells originate from the germinal zones of the brain, express precursor/progenitor-specific antigenic markers, and migrate throughout the CNS. Precursor/progenitor cells retain their mitotic ability throughout their migratory period. Once the progenitor cell reaches its target destination, it loses its migratory and mitotic capacity and takes on the post-migratory pro-oligodendrocyte phenotype. The post-migratory pro-oligodendrocyte down regulates the expression of many cell-surface oligodendrocyte progenitor cell markers and begins expressing surface sulfatides and glycolipids (Pfeiffer et al., 1993; Baumann and Pham-Dinh, 2001). At this stage, the cell develops into an immature oligodendrocyte and continues to mature into a myelinating oligodendrocyte as other specific markers are acquired.

Figure 1.1



Migratory Precursor	Migratory Progenitor	Post-Migratory Pro-Oligodendrocyte	Immature Oligodendrocyte	Myelinating Oligodendrocyte
A2B5	A2B5	A2B5	DM-20	DM-20/PLP
Nestin	NG2	NG2	CNP	CNP
PDGFαR	PDGFαR	PDGFαR	O4	O4
DM-20	DM-20	DM-20	O1	O1
CNP	CNP	CNP	GalC	GalC
PSA-NCAM		O4		MBP
				MAG
				MOG

Figure 1.1 Illustration depicting the various stages of oligodendrocyte development and the proteins associated with each of these maturational stages.

*Figure Adapted from Pfeiffer et al., 1993; Baumann & Pham-Dinh, 2001; Fox, 2004.

Functional Roles of Mature Oligodendrocytes

The functional roles of mature oligodendrocytes include: 1) the development of the axonal myelin sheath which enhances the speed of action potential propagation, 2) aiding in the diametric growth of CNS axons (Colello et al., 1994; Xu et al., 1996; Umemori et al., 1999; Osterhout et al., 1999), and 3) neuronal maintenance and survival (Pfeiffer et al., 1993; Baumann & Pham-Dinh, 2001; Wilkins et al., 2003). The oligodendrocyte has also been shown to inhibit neuronal regenerative capacity via the expression of neurite outgrowth inhibitory proteins (Baumann & Pham-Dinh, 2001).

Proteins Involved in Myelination

Most neurons in the vertebrate central nervous system have axons that are insulated by a lipid-rich layer, which wraps in segments of 1-2 mm along their length. This layer, which segregates the conducting axoplasm from the extracellular fluid, is known as the myelin sheath, and is produced and/or laid down by oligodendroglial cells. The myelination process is heavily dependent on proteins that initiate and maintain axonal-oligodendrocyte interactions **(Table 1.1)**.

The myelination process can be broken down into three distinct stages: 1) ***Initiation***: the recognition of target axons for myelination, 2) ***Compaction***: the extrusion of the oligodendrocytes cytoplasm leading to the close apposition of the cytoplasmic leaflets of the plasma membrane of the myelinating cell, and 3) ***Maintenance***: the preservation of the structural integrity of the myelin sheath.

One protein that has been implicated in the initiation of the myelination process is myelin-associated glycoprotein (MAG; 64, 69, & 100 kDa). Past studies have shown that

Table 1.1 Proteins Involved in the Myelination Process	
Initiation	Myelin-Associated Glycoprotein (MAG) Fyn Tyrosine Kinase (Fyn PTK) Neurofascin (Fibronectin type III)
Compaction	Myelin Basic Protein (MBP) Proteolipid Protein (PLP) / DM20 Myelin-associated Oligodendrocyte Basic Protein (MOBP) Oligodendrocyte Specific Protein (OSP) Connexin-32/45 (Cx32/45) Tetraspan-2 UDP-Galactose: Ceramide galactosyltransferase (CGT)
Maintenance	2'-3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase) Oligodendrocyte-myelin glycoprotein (OMgp) Myelin/Oligodendrocyte Gylcoprotein (MOG) Myelin/Oligodendrocyte Specific Protein (MOSP)
Other Proteins	RIP Antigen

Table 1.1 The myelination process can be broken down into three distinct stages: 1) ***Initiation***, 2) ***Compaction***, and 3) ***Maintenance***. This table lists a number of proteins that have been implicated in the various stages of the myelination process.

MAG is a heavily glycosylated integral membrane protein that is synthesized by ribosomes on the rough endoplasmic reticulum. Its early appearance and placement in periaxonal membranes of myelin internodes suggests a role in the axon-myelin initiation process (Poltorak et al., 1987; Trapp, 1990; Pfeiffer et al., 1993; Umemori et al., 1999). It has been suggested that MAG may be involved in the recognition of nonmyelinated axons by oligodendrocyte processes (Li et al., 1998). MAG, itself, is the ligand involved in the adhesion of the oligodendrocyte process with the neuronal axon. Once the MAG ligand adheres to the axon, it is stimulated to activate the Fyn protein tyrosine kinase (Fyn PTK) (Umemori et al., 1999). Fyn PTK works through a signaling pathway to stimulate transcription of the myelin basic protein (MBP) gene **(Fig. 1.2)**.

MBP is an intracellular membrane protein, which is synthesized by free polyribosomes in the outer processes of oligodendrocyte myelin. This highly charged protein comprises 30-40% of total CNS myelin protein (14-21 kDa isoforms in the rat). It has been suggested that as a result of post-translational methylation and its high affinity for membranes, MBP mediates the close apposition of myelin cytoplamic leaflet membranes thus forming the compact structure of the axonal ensheathment (Pfeiffer et al., 1993; Brady et al., 1999; Wegner, 2000; Baumann & Pham-Dinh, 2001). While most proteins responsible for development and/or myelinogenesis are down-regulated after the active myelination period, MBP expression is sustained throughout adulthood (Wegner, 2000).

Proteolipid protein (PLP; 30 kDa) is the most abundant protein in oligodendrocyte myelin comprising nearly 50% of the total protein mass. PLP and its spliced isoform DM20 (26 kDa) are hydrophobic integral membrane proteins synthesized by perinuclear

Figure 1.2

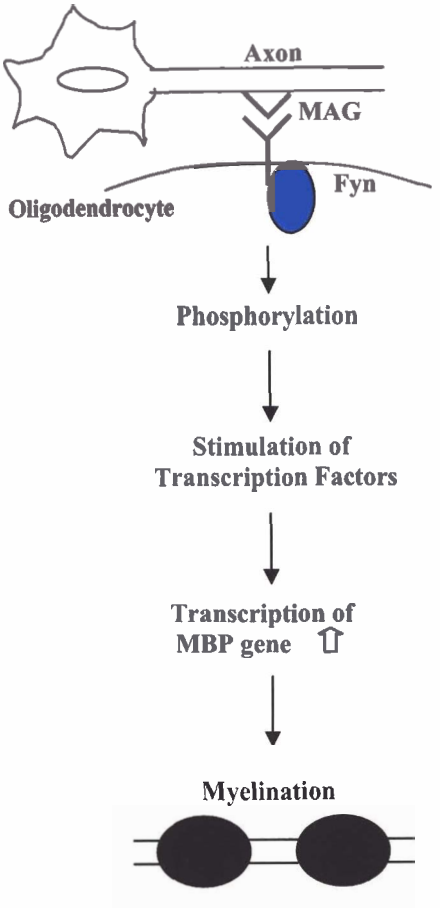

Axon
MAG
Fyn
Oligodendrocyte
Phosphorylation
Stimulation of
Transcription Factors
Transcription of
MBP gene ⇧
Myelination

Figure 1.2 Illustration of the signaling pathway in which Fyn PTK stimulates transcription of the myelin basic protein (MBP) gene.

* Figure adapted from Umemori et al., 1999.

ribosomes on the rough endoplasmic reticulum. These proteins function in oligodendrocyte development, compaction of extracellular myelin membrane leaflets, and axonal integrity (Trapp, 1990; Pfeiffer et al., 1993; Wegner, 2000). DM20 is expressed by oligodendrocytes throughout their differentiation process. Upon full maturation, the myelinating oligodendrocyte expresses both DM20 and PLP during the compaction stage of myelin formation. PLP is found at the intraperiodic and major dense lines of myelin, functioning to stabilize the ensheathment by acting as a zipper for the independent sheets of myelin (Boison et al., 1995; Klugmann et al., 1997; Baumann & Pham-Dinh, 2001).

Myelin-associated oligodendrocyte basic protein (MOBP; 8-12 kDa), a small, highly basic protein, is found in the body of the immature oligodendrocyte and moves distally into the major dense lines of myelin processes as myelination proceeds (Yamamoto et al., 1994; Baumann & Pham-Dinh, 2001). It is thought that MOBP plays a similar role to MBP in the compaction process due to the timing of its distal migration and location within the oligodendrocyte/myelin processes (Holz & Schwab, 1997).

Oligodendrocyte specific protein (OSP; 22 kDa), the third most abundant protein in CNS myelin, functions as a tight junction protein forming parallel-array strands in CNS myelin (Bronstein et al., 1997; Gow et al., 1999, Baumann & Pham-Dinh, 2001). Due to the similar tetraspan membrane structure to PLP and its location within myelin as arrayed tight junctions, it is believed this protein plays a significant role in myelin compaction.

Connexin-32 (Cx32) and Connexin-45 (Cx45), known gap junction proteins, have been found to be expressed by oligodendrocytes (Scherer et al., 1995; Dermietzel et al.,

1997; Li et al., 1997; Kunzelmann et al., 1997). These proteins play possible dual roles in compaction and maintenance of the myelin sheath (Baumann & Pham-Dinh, 2001).

2'-3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase; 46 & 50 kDa), a basic, peripheral myelin membrane protein located on the cytoplasmic face of the oligodendrocyte cell body and processes, comprises roughly 4% of the total mass of oligodendrocyte myelin protein. CNPase, which is translated by free polysomes and expressed throughout oligodendrogenesis, is found on the cytoplasmic side of the plasma membrane of non-compacted myelin, within the paranodal loops, and at the periaxonal membrane (Trapp, 1990). Its location within the cell suggests that CNPase may have a general role in the maintenance of membranes that demarcate cytoplasmic channels within the myelin internode and plasma membranes of the oligodendrocyte. The enzymatic activity of CNPase *in vitro* serves to hydrolyze 2'3'-cyclic nucleotides into their corresponding 2'-nucleotide derivatives; however its *in vivo* function within the oligodendrocyte remains elusive since 2'3'-nucleotides have yet to be detected in the brain (Trapp, 1990; Baumann & Pham-Dinh, 2001; Popko, 2003).

Myelin/oligodendrocyte glycoprotein (MOG; 25 kDa), an integral membrane protein synthesized by ribosomes on the rough endoplasmic reticulum, is expressed at a later time period in the myelination process and is a marker for oligodendrocyte maturation **(Fig. 1.1)**. Its location on oligodendrocyte processes and outermost lamellae of the compact myelin sheath suggests that it plays a role in myelin maintenance. MOG has been found to be the most encephalitogenic amongst myelin proteins suggesting a major role in multiple sclerosis and other autoimmune demyelinating diseases (Piddlesden & Linington, 1990; Baumann & Pham-Dinh, 2001).

Some other minor oligodendrocyte proteins have been found to play a role in the myelination process. Oligodendrocyte-myelin glycoprotein (OMgp; 120 kDa) is attached to CNS myelin through a glycosyl phosphatidylinositol and is limited to the paranodal regions, thus suggesting a role in myelin maintenance (Baumann & Pham-Dinh, 2001). Myelin/oligodendrocyte specific protein (MOSP; 48 kDa) is found on the extracellular surfaces of the myelin processes of the oligodendrocyte, again suggesting a role in maintenance of the myelin sheath (Dyer et al., 1991; Baumann & Pham-Dinh, 2001). Other proteins thought to play specific roles in oligodendrocyte myelination, such as the RIP antigen, have been suggested; however their exact roles are unclear at this time.

Proteins Involved in Axonal Diametric Growth

Axons undergo considerable modifications in their diametric growth throughout CNS development. Upon arrival at their target destination, axons expand radially 3 to 10 fold to achieve a diameter sufficient for rapid, efficient action potential conduction (Sanchez et al., 2000). Oligodendrocyte presence has been shown to influence the caliber of mature axons and their radial growth (Colello et al., 1994) by up to 40%. Both oligodendrocyte proteins and neuronal proteins, whose expression levels are dependent upon the presence of oligodendrocytes, are believed to play significant roles in axonal diametric growth **(Table 1.2)**.

Regulation of axon diameter has been linked to the accumulation of neurofilaments, composed of three subunits, NF-L (70 kDa), NF-M (150 kDa), and NF-H (200 kDa), within the axon in the presence of oligodendrocytes (Xu et al., 1996; Starr et al., 1996; Sanchez et al., 2000). Sanchez et al. (1996) have shown that oligodendrocyte presence,

Table 1.2 Proteins Involved in Diametric Axonal Growth	
Spacing	Axonal Neurofilament-L, -M, & -H (NF-L,M,H)
Control & Maintenance	Myelin-associated Oligodendrocyte Basic Protein (MOBP)

Table 1.2 This table lists oligodendrocyte proteins and neuronal proteins, whose expression levels are dependent upon the presence of oligodendrocytes, that are believed to play significant roles in axonal diametric growth.

not myelination, induces full axonal radial growth through a signaling mechanism that triggers local accumulation and reorganization of the neurofilament network. Radial growth is controlled by the state of neurofilament phosphorylation. Neurofilaments, within myelinated axonal segments, contain 40-50 Lys-Ser-Pro amino acid sequence repeats. Neurofilament spacing is directly correlated to the level of phosphorylation of the serines in the amino acid repeats (Xu et al., 1996). Repulsion between the adjacent filaments, as a result of high levels of negatively charged phosphates, leads to a wider spacing arrangement and resulting axonal expansion. Thus, an increase in neurofilament phosphorylation induced by extrinsic oligodendrocyte signals leads to increased axonal radial growth.

Although it is unclear how the radial growth of axons is limited, Sadahiro et al. (2000) have found that myelin-associated oligodendroctye basic protein (MOBP) plays a role in controlling and/or limiting axonal diametric growth and also works to maintain the cylindrical shape of the axon.

Proteins Involved in Neuronal Maintenance & Survival

Another important contribution of oligodendrocytes to the function of the CNS is in the maintenance and survival of neurons **(Table 1.3)**. It has been demonstrated that oligodendrocytes express the iron mobilization protein, transferrin, which acts as a trophic factor for both neurons and astrocytes (Espinosa de los Monteros et al. 1989). PLP, through glial-axonal communication, has also been shown to maintain axonal integrity and function (Baumann & Pham-Dinh, 2001). Studies demonstrated that in the absence of PLP/DM20 protein expression, axonal abnormalities referred to as 'spheroids'

Table 1.3 Proteins Involved in Neuronal Survival	
Survival	Transferrin Proteolipid Protein (PLP) Glial cell Line-Derived Neurotrophic Factor (GDNF)

Table 1.3 This table lists oligodendrocyte proteins that are believed to play significant roles in the maintenance and survival of neurons within the CNS.

develop (Griffiths et al., 1998), while a near normal myelin sheath is laid down by the oligodendrocytes. The 'spheroids,' composed of numerous mitochondria and other membranous bodies, interrupt axonal transport resulting in severe neuronal abnormalities and axonal degeneration.

Wilkins et al. (2003) show that oligodendrocytes release trophic factors promoting neuronal survival. Their research provides evidence that glial cell line-derived neurotrophic factor (GDNF) is produced by differentiated oligodendrocytes and works to promote neuronal survival and axonal integrity. Thus, oligodendrocytes further contribute to the functioning of the CNS through their regulation of protein expression.

Inhibition of Axonal Regenerative Capacity

Beyond the initiation, compaction, and maintenance of the myelin sheath, as well as neuronal survival, proteins expressed by oligodendrocytes can influence neurite outgrowth. Lesioned axons fail to completely re-establish connections within the mammalian nervous system following insult and/or injury. It is thought that oligodendrocytes play a significant role in limiting axonal regeneration through the expression of specific inhibitory proteins (Blight, 1998; Qiu et al., 2000; Bandtlow & Schwab, 2000) **(Table 1.4)**. The inhibitory role of oligodendrocytes may also serve as boundary and guidance tools for late-developing CNS tract neurite outgrowth (Baumann & Pham-Dinh, 2001).

NI-35 and NI-250 proteins, now commonly referred to as NOGO proteins, are highly expressed membrane-bound proteins in oligodendroctyes and their myelin processes. These transmembrane proteins have been identified as inhibitors of axonal re-growth and

Table 1.4 Proteins Involved in Inhibition of Axonal Growth	
Inhibition	NI-35/250 (NOGO) Myelin-Associated Glycoprotein (MAG)

Table 1.4 This table lists proteins expressed by oligodendrocytes that can influence neurite outgrowth. It is believed that oligodendrocytes play a significant role in limiting axonal regeneration through the expression of specific inhibitory proteins. The inhibitory role of oligodendrocytes also serves as guidance tools for late-developing CNS neuronal axon sprouting.

neural plasticity in pathological states (Caroni & Schwab, 1988). MAG has also been implicated as a CNS axonal inhibitory protein (McKerracher et al., 1994; Li et al., 1996). Li et al. (1996) have shown that the presence of MAG *in vitro* causes growth cone collapse in migratory regenerating axons following insult.

Oligodendrocyte Regulation of Neuronal and Astrocytic Proteins

The above section describes a number of known oligodendrocyte functions and the roles in which oligodendrocyte protein expression play in fulfilling those numerous functions. However, our current understanding of oligodendrocyte-derived and associated protein expression is still limited. It is known that the pattern of expression of select neuronal proteins is influenced by oligodendrocyte cell-cell interactions (Xu et al., 1996; Starr et al., 1996; Einheber et al., 1997; Sanchez et al., 2000; Baumann & Pham-Dinh, 2001). The presence of myelinating glial cells in the CNS is critical for the expression of numerous selected neuronal proteins. For example, the expression level of two axonal membrane proteins, Caspr and Contactin, has been shown to be elevated during glial ensheathment (Einheber et al., 1997). The expression of neuronal proteins that are regulated by oligodendrocyte presence, in part, characterize a neuron's morphology and function. There are many neuronal proteins that are affected by oligodendrocyte presence that have yet to be characterized.

Unlike the neuronal-oligodendrocyte interactions described above, to our knowledge, there are no reported studies of proteins produced by astrocytes whose expression patterns are regulated by, or dependent upon, oligodendrocytes. Nevertheless, this would seem likely since the converse is true, in that, astrocytes have been shown to be crucial to

oligodendrocyte development. For example, it has been suggested that astrocytes in the developing optic nerve respond to the electrical activity of axons by expressing platelet-derived growth factor (PDGF) (Barres & Raff, 1993). PDGF has been shown to stimulate oligodendrocyte progenitor cells to proliferate; therefore, release of this factor by astrocytes could account for the proliferation of these cells seen within the developing optic nerve. Likewise, it has been shown that mice carrying a null mutation to the astrocytic gene, glial fibillary acidic protein (GFAP), display abnormal myelination (Liedtke et al., 1996). Together, these studies suggest that cell-cell interactions between astrocytes and oligodendrocytes exist and are crucial for normal myelination to proceed. It is therefore, reasonable to assume that oligodendrocytes are capable of regulating astrocytic protein expression.

In Chapter Two, we describe the methodology that allowed us to identify, *in vivo,* other proteins solely expressed by oligodendroctyes, as well as proteins expressed by neurons and astrocytes that are influenced by the presence of oligodendrocytes.

Chapter Two

Materials and Methods

The following is a detailed description of the experimental model system we utilized in an attempt to elucidate the complete repertoire of oligodendrocyte-regulated proteins.

Explanation of Experimental Model System

In previous studies, our lab had adapted the technique of X-irradiation to selectively eliminate oligodendrocyte progenitor cells from part of the rat retinofugal pathway and, by virtue of their absence, evaluated the influence of these cells on the development of a CNS fiber pathway (Colello et al., 1994; Colello & Schwab; 1994). In particular, we targeted the developing optic nerves of the rat retinofugal pathway for irradiation treatment for the following reasons: 1) oligodendocyte progenitor cells within the optic nerve, which are derived from an active prenatal migration (Small et al., 1987; Nishiyama et al., 1996), remain mitotically active into the postnatal period and are, therefore, susceptible to irradiation; 2) irradiation treatment can be confined to a specific region of the retinofugal pathway, i.e. the optic nerve, sparing the neurons of those fibers and their target areas, and 3) by restricting the treatment to one side of the brain we could compare, in the same animal, the normal development of one optic nerve with that of the X-irradiated optic nerve, which develops in the absence of oligodendrocytes.

The effectiveness of the X-irradiation treatment in preventing oligodendrocyte development in the optic nerve was first made apparent by the completely translucent and thin appearance of the X-irradiated nerve compared to that of the normal nerve at P15

(Fig. 2.1 B). This observation was verified at the EM level by the conspicuous absence, in the X-irradiated nerve, of myelin ensheathment (compare P15 normal nerve in **Fig. 2.2 A** versus P15 irradiated nerve in **Fig. 2.2 B)**. At the ultra-structural level, these P15 axons of the X-irradiated nerve appeared intact and resembled perinatal axons with the cytoskeletal elements and the axonal membrane intact and clearly delineated. The nerves undergo a postnatal period of delayed myelination, which can be seen by the presence of myelinated axons within the optic nerves of animals that were irradiated at P 0, 2, and 4 and left to survive to P28 **(Fig. 2.2 C)**. These observations suggested that this delayed myelination of the irradiated nerve may be the result of an active migration of progenitor cells into the postnatal X-irradiated nerve. This was confirmed by immunostaining X-irradiated optic nerves at the time of this delayed myelination (P12-P20) for PDGFα receptor, a marker selective for oligodendrocyte progenitor cells (Nishiyama et al., 1996), and for myelin basic protein, a marker specific for differentiated oligodendrocytes (Omlin et al., 1982) **(Fig. 2.2 D)**. Moreover, an examination of the cell types within these nerves, using a culture assay, revealed the complete absence of oligodendrocyte progenitor cells. However, from P14 onwards progenitor cells appeared within these nerves, first in the chiasmatic region of the nerve and, later, in regions of the nerve near the eye. The results from these experiments provided us with evidence that oligodendrocyte progenitor cells were migrating into these nerves from the chiasm similarly to the situation in normal development.

Figure 2.1

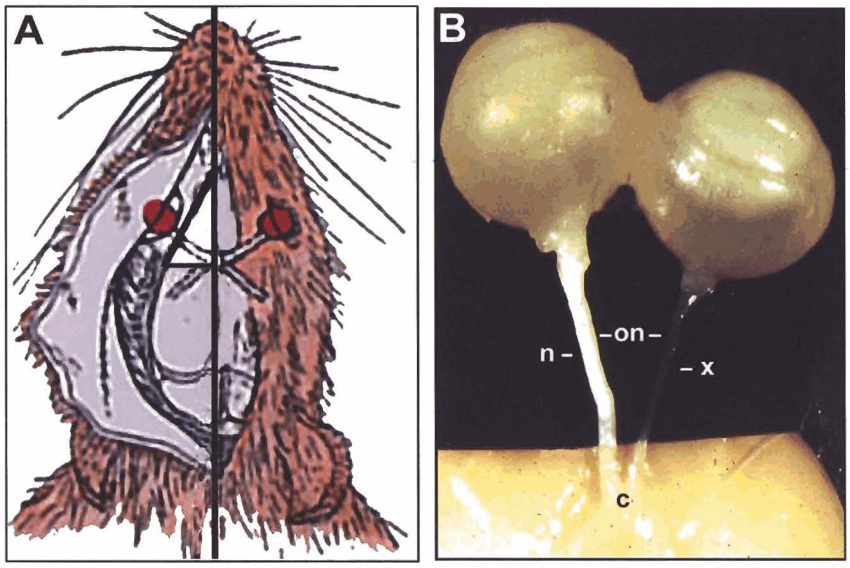


Figure 2.1 Unilateral X-irradiation eliminates oligodendrocytes from one optic nerve.

(**A**) Illustration showing a dorsal view of the area of the rat head exposed to the X-irradiation treatment (triangle) in relation to the position of the underlying optic nerves.

(**B**) Ventral view of the eyes, optic nerves (on) and chiasm (c) of a P15 rat that was unilaterally X-irradiated at PO, P2 and P4. The treated nerve (x) is myelin-free while the nerve on the left (n) is untreated and, therefore, myelinated.

Figure 2.2

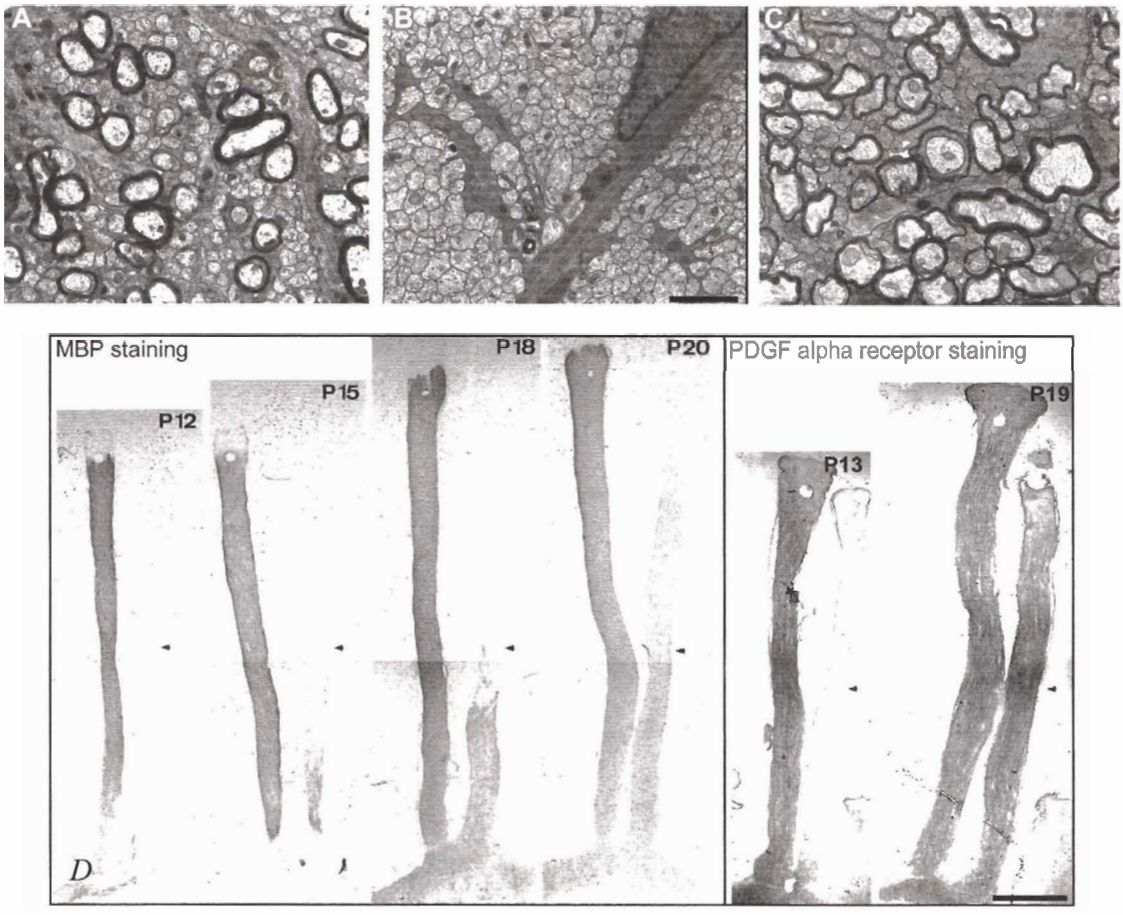

A
B
C
MBP staining
P12
P15
P18
P20
D
PDGF alpha receptor staining
P13
P19

Figure 2.2 X-irradiation treatment selectively effects oligodendrocytes and myelin formation. X-irradiated nerves go through a period of "delayed" myelination as a result of the migration of a second cohort of oligodendrocyte progenitor cells into these optic nerves.

Electron micrographs of the P15 normal (A), P15 X-irradiated (B) and P28 X-irradiated optic nerves (C). Longitudinal sections (D) through the optic nerves of P12-P20 rats treated unilaterally with X-irradiation at PO, P2 and P4. Compare the progress of delayed myelination on the irradiated side (arrowheads) to the extent of myelination on the untreated side as shown with antibodies to MBP and PDGFα receptor. Note that within the X-irradiated optic nerve, the gradient of delayed myelination begins at the chiasm (bottom of figure) and moves towards the eye (top of figure). Scale = 2mm.

Materials

Lewis albino rats were used in this study. Ammonium bicarbonate and spectrometric grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Iodoacetamide, DL-dithiothreitol, potassium ferriscyanide, sodium thiosulfate, formic acid, and calcium chloride were all obtained from Sigma Chemical Co. (St. Louis, MO). HPLC grade water was obtained from JT Baker (Phillipsburg, NJ). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI).

X-irradiation Treatment

The selective elimination of optic nerve oligodendrocyte progenitor cells in each newborn Lewis rat pup of a litter was achieved through unilateral exposure to x-irradiation treatment at P0, P2, and P4, which represents the period of oligodendrocyte progenitor cell proliferation (Small et al., 1987; Skoff, 1978; Skoff et al., 1980). P0 rat pups were anesthetized by hypothermia for approximately 5 minutes and placed in a plastic support tray made from an emptied cover slip box. Their entire bodies were covered by a 1" x 3" lead shield. To ensure unilateral destruction of the oligodendrocytes of one retinofugal pathway, a small centrally located triangular slit was cut into the shield overlying the surface of the head corresponding to the anatomical location of the right optic nerve. Efforts were made to ensure that neither the retinas nor the optic chiasms were exposed to the x-irradiation treatment. The rat pup was placed 20 cm from an x-ray source set at 45 kV and 30 mA and was administered one 1666 rad dose of x-rays. This process was repeated at P2 and P4 so as to deliver a total dose of 5000 rads. Following

the irradiation treatment, the pup was left to recover on a warming pad set to 37 degrees Celsius and then returned to its cage.

Dissection, Tissue Homogenization, & 2D-PAGE

At P14, both the normal and x-irradiated optic nerve of each pup were removed and prepared for 2-D gel electrophoresis. This process was also repeated for the P28 x-irradiated rat pups as well. The rat pups aged P14 or P28 were anaesthetized with 75-100 µl of Euthasol, perfused with phosphate-buffered saline, and the complete length of both optic nerves were dissected out. The cuts were made 1 mm from the optic chiasm and 1 mm behind the optic cup. The nerves were then pooled from a total of 10+ pups forming two separate groups: x-irradiated and normal optic nerves, and prepared for 2D polyacrylamide gel electrophoresis (2D-PAGE) by homogenization in SDS boiling buffer minus reducing agents with protease inhibitors. All homogenized samples were quantified with a bicinchoninic acid (BCA) protein assay to ensure that equal quantities of proteins (45µg) were loaded into each 2-D polyacrylamide gel. We found that the optic nerves of one litter of P14 pups contain enough protein to generate two gels. To obtain an accurate picture of the protein profile found within each optic nerve sample (P14 normal, P14 x-irradiated and P28 x-irradiated), while accounting for the slight variability in gel running, we generated a total of 4 gel sets for each group.

Standard isoelectric focusing 2D-polyacrylamide gel electrophoresis (IEF-2D-PAGE), a process for resolving complex mixtures of proteins with isoelectric points in the pH range of 3.5-10 and varying molecular weight, and NEPHGE-2D-PAGE, a process for resolving proteins with isoelectric points in the pH range >9, were performed according

to the method of O'Farrell (1975) by Kendrick Labs (Madison, WI). Briefly, the proteins in a sample were first denatured by dissolving in SDS buffer. A total of 45 μg of the denatured protein sample was loaded to the top of each gel run. For IEF-2D-PAGE, isoelectric focusing was carried out using glass tubes of inner diameter 2.0 mm using 2.0% pH 3.5-10 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ) for 9600 volt-hours. NEPHGE-2D-PAGE, using 1.5% pH 3.5-10 and 0.25% pH 9-11 ampholines (LKB Instruments, Baltimore, MD), were carried out at 200 volts for 12 hours. Fifty ng of purified internal standards, tropomyosin (M_r 33,000 and pI 5.2) and lysozyme (MW 14,000; pI 10.5-11), were added to each sample as reference markers and labeled with an arrow on the stained gel. Isoelectric focusing (IEF) was carried out overnight.

After a brief equilibration (~10 minutes) in SDS buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M tris, pH 6.8), the tube gel was sealed to the top of a stacking gel overlaying a 10% acrylamide slab gel. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards to the agarose sealing the tube gel to the slab gel: myosin (220,000 kDa), phosphorylase A (94,000 kDa), catalase (60,000 kDa), actin (43,000 kDa), carbonic anhydrase (29,000 kDa), and lysozyme (14,000 kDa). These standards appear as horizontal lines on the silver stained 10% acrylamide slab gels. SDS gel electrophoresis was carried out for 4 hours at 12.5 mA/gel followed by special-silver staining. Duplicate gels were run for each sample preparation. The stained gels were then dehydrated and sealed in transparent cellophane sheets, with the acid end to the left, for further analysis. A total of 8 gels were produced for the experimental nerve group and 8 gels were produced for the control nerve group.

Analysis of 2D-Gels

Recent publications have compared multiple computer-assisted image analysis software packages. Rosengren et al. (2003) found two programs, Progenesis and PDQuest, to be of equal analytical power and quality. When comparing manual analysis to computer-assisted software analysis, it has been shown that spot detection accuracy differs. Manual methods tend to underestimate the number of quantified spot differences; where computer programs tend to overestimate the number of quantified spot differences (Rosengren et al., 2003). Using both methods leads to the most accurate and reliably quantified data.

Manual Comparison

We initially conducted a manual analysis of the gel groups (P14 normal, P14 x-irradiated, P28 x-irradiated) by illuminating the silver stained 2-D gels with a lightbox. Spots displaying a pattern of expression consistent with oligodendrocyte-associated proteins were demarcated and numbered on an overlying transparency. Following this comparison, the 2D gels were digitally scanned using a GS-800 (Bio-Rad, Auburn, CA) at the University of Virginia. The scanned images were uploaded onto Adobe Photoshop and a further manual comparison was done by separating the 2D gels into sections and visually comparing computer enlarged gel images.

Digitized Gel Analysis

In conjunction with a detailed manual analysis, a computerized analysis of the 2-D gels was undertaken, which included automatic spot finding and quantification, automatic

background subtraction, and automatic spot matching. Specifically, gels were digitized with a Bio-Rad GS-800 scanner and analyzed with the Discovery Series™ PDQuest 2D-Gel Analysis software (Version 7.3.1, Bio-Rad). Once the digitized images were uploaded into the analysis software, the digital gels were cropped and prepared for spot detection. Following the detection of spots, the digitized images were filtered and smoothed to clarify the spots.

Two parameters, spot volume and spot quality, were used by PDQuest to quantitatively compare the protein spots in the digitized gels. Those spots that were unable to be automatically defined accurately by the software program were manually defined with the spot-boundary tool. The total staining intensity in a gel image was used to normalize spot density because of inconsistent silver-staining methods. Only those spots representing a substantial 2 fold decrease of protein expression in the sample gel (P14X-irradiated optic nerves) as compared to the reference gel (P14 normal optic nerves) were further analyzed with mass spectrometry. The MW and pI values for each spot were determined from algorithms applied to the reference image.

The following techniques were used to prepare the selected proteins for mass spectrometric analysis. It should be noted that we used many of these techniques in our initial attempts at identifying these proteins in collaboration with the VCU Proteomic Core Facility. However, these attempts were unsuccessful, therefore, protein spots were submitted to both Columbia University (MALDI-TOF) and Stanford University's (ESI-LC-MS/MS) Mass Spectrometry Facilities. The spots identified in the Result's Section of this thesis were the consequence of their efforts.

In-Gel Digestion of Special Silver Stained Proteins for MALDI-TOF Analysis

For MALDI-TOF, gels were prepared for digestion by staining with special-silver stain. Spots of interest were excised and placed in separate tubes where they were destained according to Gharandi et al. (1999). The samples were then dehydrated with 200 μL acetonitrile (ACN). The ACN was removed and the gel dried for 30 minutes in a Speed-Vac concentrator. Rehydration of the gels was performed with 0.01 μg modified trypsin and 0.1 μg Lys-C in the minimum amount of 0.025M Tris, pH 8.5. These solutions were incubated overnight at 32°C. When digestion was complete, the peptides were extracted with 2X 50μl 50% ACN / 2% TFA, dried, and resuspended in matrix solution. Matrix solution was prepared by making a 10 mg/ml solution of 4-hydroxy-α-cyanocinnamic acid in 50% ACN/ 0.1% TFA. The dried peptides were dissolved in 3 ml of the matrix solution with the internal standards, angiotensin and bovine insulin. A 0.7 ml aliquot of this solution was spotted onto a sample plate and allowed to dry. After washing the spot twice with water, MALDI-TOF mass spectrometric analysis was performed.

In-Gel Digestion of Special Silver Stained Proteins for LC-ESI-MS/MS Analysis

For liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), gels were prepared for digestion by rinsing with HPLC-grade water. Spots of interest were excised with varying sized tissue punchers in an attempt to minimize the volume of the background gel, chopped into 1 mm x 1 mm cubes, and transferred into separate low retention/siliconized microcentrifuge tubes.

Reduction/Alkylation

The gel particles were first washed with 100 µl of HPLC water for 5 minutes and spun down. Following the removal of the liquid, 100 µl of ACN was added and placed on a mixer for 15 minutes until the gel particles were shrunken together. The particles were spun down, the liquid was removed, and the samples were dried by vacuum centrifugation. The gel particles were then swelled in 50 µl 10 mM dithiothreitol/100 mM ammonium bicarbonate and incubated for 30 minutes at 56° C with shaking to reduce protein. After spinning the gel pieces down and removing the liquid, they were again shrunk with 100 µl of ACN for 15 minutes with shaking. The ACN was then replaced by 55 mM Iodoacetamide/100 mM ammonium bicarbonate and incubated for 20 minutes in the dark at room temperature. Following spinning and removal of the 55 mM iodoacetamide the gel pieces were again shrunk with 100 µl of ACN, spun down, and dried by vacuum centrifugation.

Tryptic Digestion

The gel particles were rehydrated with digestion buffer (50 mM ammonium bicarbonate/5 mM calcium chloride/12.5 ng/µl sequencing grade modified trypsin) and placed in an ice bucket (4° C) for 30-40 minutes to allow for absorption. The remaining supernatant was removed and replaced by 25 µl of buffer without sequencing-grade modified trypsin and set in an oven overnight at 37° C.

Peptide Extraction

For LC-ESI-MS/MS analysis, tryptic peptides must be extracted from the gel particles. To accomplish this, 15 µl of 25 mM ammonium bicarbonate was added and incubated at 37° C for 15 minutes with shaking. The particles were then spun down and 100 µl of ACN was added and incubated at 37° C for 15 minutes with shaking. The gel particles were again spun down and the supernatant was collected and set aside. Fifty microliters of 5% formic acid was added to the gel particles and vortexed for 15 minutes at 37° C. The gel particles were spun down, 100 µl of ACN was added for 15 minutes with shaking, and spun down again. The gel particles were removed and discarded, and the extract was pooled together with the previous supernatant, dried down in a vacuum centrifuge, and frozen at -20° C until mass spectrometric analysis.

Mass Spectrometry

Both matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and liquid chromatography-electrospray ionization-quadrupole-ion trap mass spectrometry (LC-ESI-MS/MS) were used in this study. We had decided on this dual approach because these two methodologies are highly sensitive and complementary. Specifically, MALDI-TOF can be used to quickly obtain peptide mass fingerprints, is reasonably tolerant to contamination by buffers and salts, and allows for the analysis of complex mixtures at relatively low mass resolution (Hunt & Sheil, 1998; Loo et al., 1999; Zhan & Desiderio, 2003). We also employed a LC-ESI-MS/MS procedure to determine the amino acid sequence of proteins of interest because of its greater range of detection limits compared to that of MALDI-TOF (Hunt & Sheil, 1998; Loo et al., 1999; Zhan &

Desiderio, 2003). However, LC-ESI MS/MS sensitivity can be dramatically reduced by contamination from buffers, salts and cellular components. For LC-ESI-MS/MS, the frozen, extracted, un-fractionated tryptic peptides were placed in 13.4 µl of resuspension solution and subjected to ESI-LC-MS/MS. Ten microliters of the resuspended peptide fragment solution was picked up by the auto-sampler and fed to the nano-HPLC which was connected directly to a ThermoFinnigan LCQ Deca XP Plus (ThermoFinnigan, San Jose, CA) utilizing a fully automated coupling to the mass spectrometer. The ThermoFinnigan Bioworks 3.0 software controls the LC Packing nano-HPLC and all experiments.

A Data-Dependent™ acquisition cycle begins with a full MS scan; followed by a full MS/MS scan of the most intense ions selected from the preceding MS spectrum. Proteins are identified by submitting the MS and MS/MS spectra to database searches **(Fig. 2.3)**.

Immunoblotting

Immunobloting was used to confirm that a select number of the proteins identified by MS matched the expected expression pattern; that is, present in P14 normal nerve, absent in the 14 X-irradiated nerve, and reappearing in the P28 X-irradiated nerve. Briefly, 2-D gels were transblotted onto PVDF membranes (Immobilon from Millipore Corp.) Sections of the PVDF membranes that corresponded to molecular weights and pI's of the proteins of interest were excised with a scalpel. Blocking of the sectioned PVDF's was performed with 5% fat-free dry milk in TBST for one hour. Primary antibodies for each protein were applied to the membranes and left on a shaker overnight at 4°C. Membranes

Figure 2.3

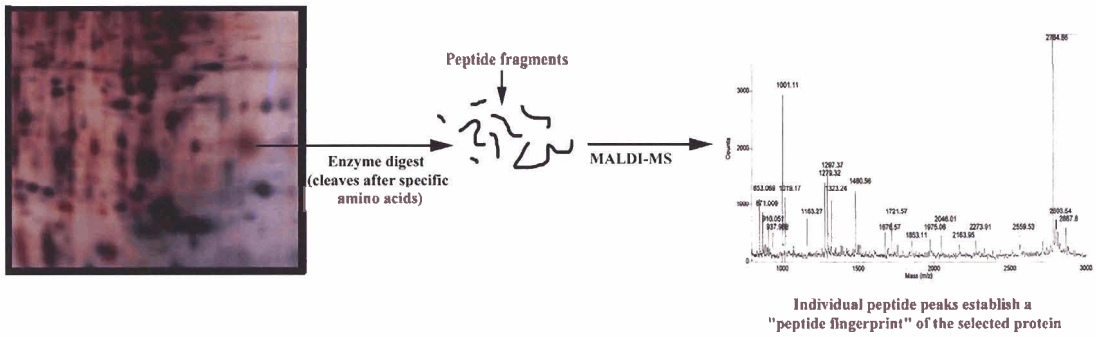

Peptide fragments
Enzyme digest
(cleaves after specific
amino acids)
MALDI-MS
Individual peptide peaks establish a
"peptide fingerprint" of the selected protein

Figure 2.3 Enzymatic digest cleaves proteins into individual peaks of specific molecular weights.
This illustration highlights the procedures used to identify proteins within our 2-D gel preparation. Using trypsin as an enzymatic digest, proteins were cleaved just downstream of the amino acids, arginine and lysine, thereby generating peptide fragments of varying molecular weights. These peptide fragments were then ionized by MALDI-MS and/or LC-ESI-MS/MS to establish a molecular weight profile of the peptide fragments that were compared with peptide fingerprint profiles found within theoretically and experimentally-derived databases, i.e. SWISSPROT, MSfit, Mascot, TurboSequest, etc.

were then washed 3 times for 10 minutes in TBST, and the secondary antibodies were diluted in phosphate buffer and applied for one hour. The PVDF sections were then washed 3 times with TBST. Films of the immunoblots were developed with a chemiluminescence detection kit (Pierce West Pico), and the protein expression pattern was recorded. PVDF sections were then stripped and restained for cyclosporin, a protein present in both the normal and X-irradiated optic nerves, to verify that the proteins were properly transferred to the PVDF membranes.

Overview

We have established a methodology to identify novel molecular players involved in the regulation of oligodendrocyte function **(Fig. 2.4)**. With this method, we will further show that these include not only oligodendrocyte-derived proteins, but also oligodendrocyte-regulated proteins that may be expressed by non-oligodendroglial cells present in the optic nerve. Additionally, we will show that the X-irradiation model described above provides a unique system in which oligodendrocytes are selectively eliminated from the optic nerve while other cells remain relatively unaffected. Moreover, by restricting the treatment to one side of the brain and then isolating the proteins present in the P14 X-irradiated and P14 normal nerve, we were able to compare from the same animal, protein profiles in optic nerves with or without oligodendrocytes. As our data demonstrates, the X-irradiation selectively eliminated oligodendrocytes. Any proteins present in the normal nerves that are now absent in the X-irradiated nerves are proteins expressed by oligodendrocytes, or could be expressed by another cell type but regulated by oligodendrocytes. Using 2-D gel electrophoresis, mass spectrometry and

Figure 2.4

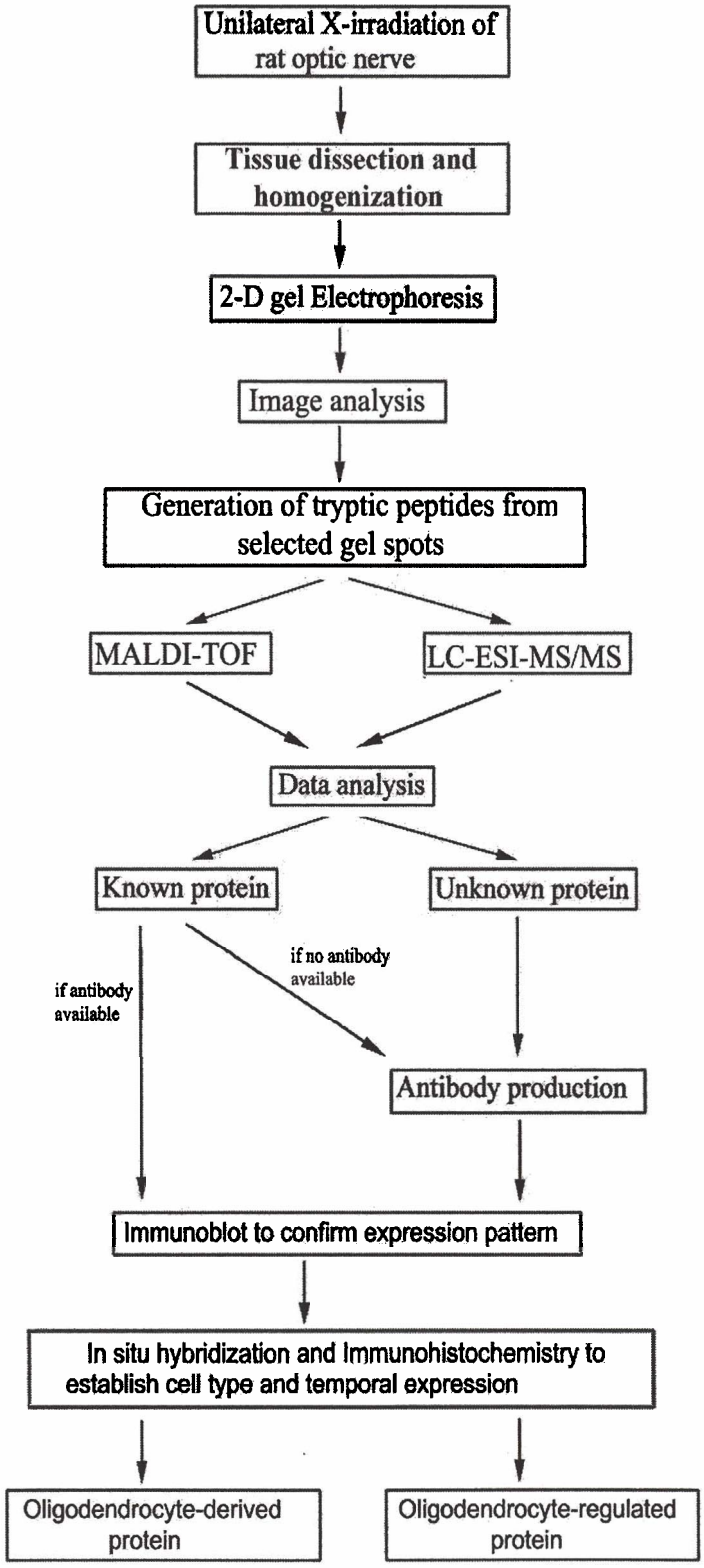

Unilateral X-irradiation of rat optic nerve
Tissue dissection and homogenization
2-D gel Electrophoresis
Image analysis
Generation of tryptic peptides from selected gel spots
MALDI-TOF
LC-ESI-MS/MS
Data analysis
Known protein
Unknown protein
if antibody available
if no antibody available
Antibody production
Immunoblot to confirm expression pattern
In situ hybridization and Immunohistochemistry to establish cell type and temporal expression
Oligodendrocyte-derived protein
Oligodendrocyte-regulated protein

Figure 2.4 Flowchart of Experimental Design
This illustration depicts the methodology used to identify novel molecular players involved in the regulation of oligodendrocyte function. These molecular players include not only oligodendrocyte-derived proteins, but also oligodendrocyte-regulated proteins that may be expressed by non-oligodendroglial cells present in the optic nerve. Further immunoblotting and immunohistochemical techniques must still be utilized to verify the identities of the proteins given by our mass spectrometric analysis and database search.

immunoblots we have identified a number of oligodendrocyte-associated proteins that were expressed in the normal P14 nerve and absent in the 14 X-irradiated nerve. Further verification that these proteins were indeed related to oligodendrocytes (and/or the processes of myelination) was obtained by their reappearance in the 2-D gel protein profile of P28.

Chapter Three

Results

We compared protein profiles found within the normal myelinating optic nerve versus the X-irradiated optic nerve to identify novel oligodendrocyte-associated proteins. These protein profiles were obtained by 2-D gel electrophoresis of P14 normal optic nerves and P14 X-irradiated optic nerves. We verified that these proteins were indeed related to oligodendrocytes and/or the process of myelination by demonstrating their reappearance in the 2-D gel protein profile of P28 X-irradiated nerves that have gone through the period of delayed myelination (refer to **Fig. 2.3**). Accordingly, by comparing 2-D gel protein profiles generated from P14 normal optic nerves and those derived from both P14 and P28 X-irradiated optic nerves, we have found a number of distinct proteins present in the normal P14 rat optic nerve, that are absent in the X-irradiated nerve at P14, and then re-appear in the X-irradiated nerve at postnatal day 28 **(Fig. 3.1)**.

Using immunoblots to 2D gels of the same protein samples as used above, we then confirmed that three of the proteins expressed in this manner were the well-characterized oligodendrocyte proteins, proteolipid protein (PLP), myelin basic protein (MBP) and myelin-oligodendrocyte basic protein (MOBP) **(Fig. 3.2)**. Likewise, we found that PDGF α receptor protein was abundantly expressed in the P14 normal nerve protein sample but absent from the P14 X-irradiated nerve protein sample, demonstrating that the X-irradiation treatment selectively eliminated oligodendrocyte progenitor cells from these

Figure 3.1

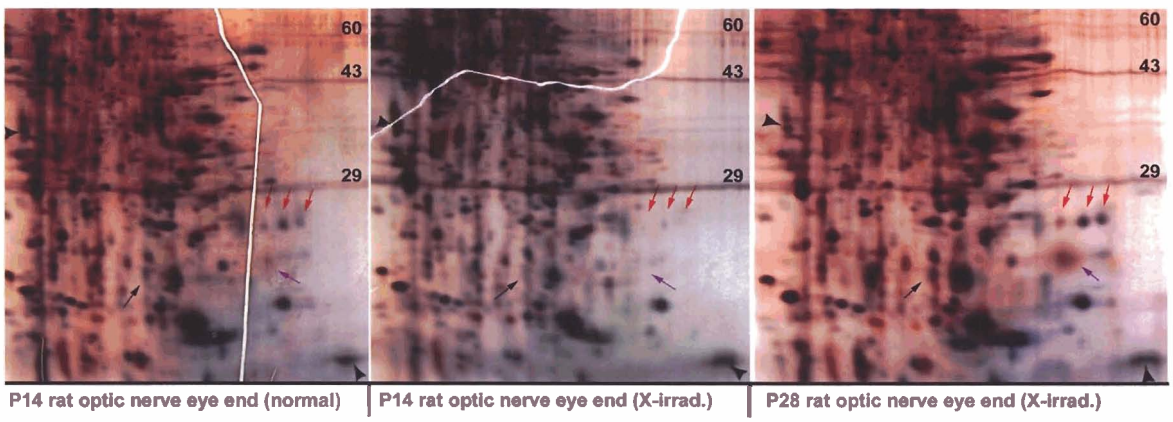

60
43
29
60
43
29
60
43
29
P14 rat optic nerve eye end (normal)
P14 rat optic nerve eye end (X-irrad.)
P28 rat optic nerve eye end (X-irrad.)

Figure 3.1 Proteomic maps derived from normal and X-irradiated optic nerves identify oligodendrocyte-associated proteins.

Silver-stained 2-D gels showing proteins present in the eye-end of P14 normal optic nerves (left) versus P14 X-irradiated optic nerves (middle) and P28 X-irradiated optic nerves (right). The colored arrows point to distinct proteins that are present in P14 normal nerves, absent in P14 X-irradiated nerves and then reappear in P28 X-irradiated nerves.

Standards: left arrowhead-Tropomyosin MW 33kD, pI 5.2; right arrowhead-Lysozyme MW 14kD, pI 10.5-11.

Figure 3.2

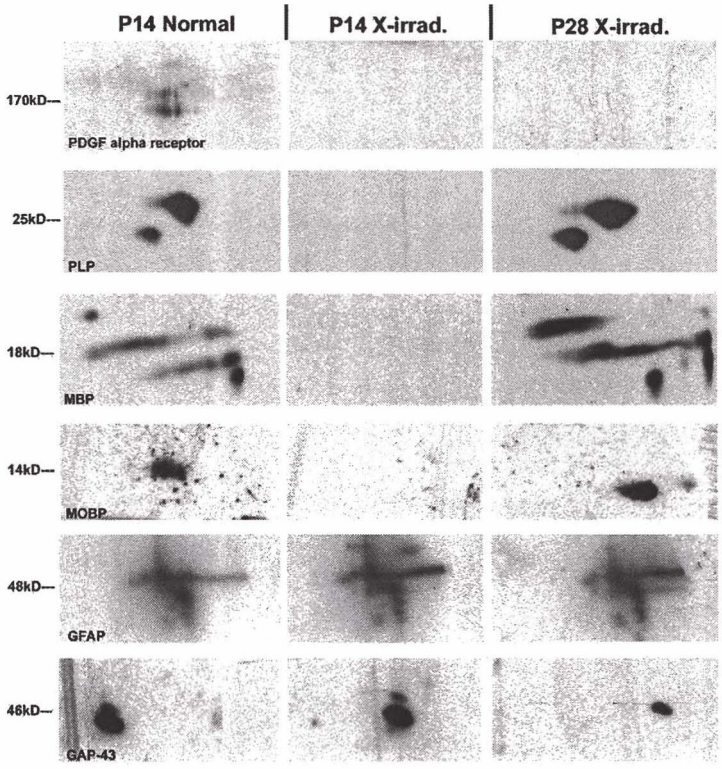

P14 Normal
P14 X-irrad.
P28 X-irrad.
170kD---
PDGF alpha receptor
25kD---
PLP
18kD---
MBP
14kD---
MOBP
48kD---
GFAP
46kD---
GAP-43

Figure 3.2 The expression of myelin proteins in the nerve is selectively and transiently affected by X-irradiation.
Immunoblots of 2-D gels demonstrating the presence or absence of specific proteins in the normal and X-irradiated optic nerve at postnatal days (P) 14 and 28. Note that the oligodendrocyte proteins are absent in the P14 X-irradiated optic nerve but re-appear in the P28 X-irradiated nerve. Whereas the expression of axonal and astrocytic proteins appears to be unaffected by the X-ray treatment.

nerves. The absence of PDGF α receptor expression in the P28 nerve sample, together with the observed expression of PLP, MBP and MOBP at this time point, suggested that all the progenitor cells that entered the irradiated nerve after P14 had differentiated into mature, myelin-forming oligodendrocytes. Furthermore, we found that the expression of the axonal and astrocytic proteins, GAP- 43 (Jacobson et al., 1986) and GFAP (Dahl & Bignami, 1976), respectively, appear to be unaffected by the X-irradiation treatment. These findings suggest that any proteins expressed in the normal P14 nerve, absent in the P14 X-irradiated nerve and re-appearing in the P28 X-irradiated nerve are very likely to be oligodendrocyte-associated proteins. Moreover, we show that even myelin proteins expressed at very low levels are detected by this method since MOBP, which represents only 0.1% of the total myelin protein (Yamamoto et al., 1994; Holz et al., 1996), was easily detected.

MALDI-MS

With this in mind, we set out to identify, by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), a further group of proteins that exhibited this characteristic pattern of expression in the 2-D NEPHGE gels shown in **Figure 3.1**. Selected protein spots, which are indicated in **Figure 3.2**, were isolated from the 2-D gels of P14 normal rat optic nerves and analyzed by mass spectrometry. In this manner, it was determined that the three proteins marked in the 2-D gels of **Figure 3.1** were the oligodendrocyte proteins, proteolipid protein (PLP) (Agrawal et al., 1977) (purple arrow in **Fig. 3.1**), phosphatidylethanolamine-binding protein (PEBP) (Frayne et al., 1997)

(black arrow in **Fig. 3.1**), and p25 (brain-specific 25-kDa protein) (Takahashi et al., 1993) (red arrow in **Fig. 3.1**).

Additionally, we found that the proteins labeled with red arrows in **Figure 3.1** displayed identical peptide spectra, suggesting that the change in pI between these proteins may be a post-translational modification of p25. More significantly, our initial survey of these 2-D gels detected a protein, which displayed the characteristic pattern of expression of oligodendrocyte proteins in our model, but had never been shown to be expressed by these cells. Specifically, this protein, identified by MALDI-MS as cofilin, was expressed in the normal P14 nerve, absent in the X-irradiated P14 nerve, and reappeared in the P28 X-irradiated optic nerve. To confirm this result, we obtained a commercially available antibody for cofilin and ran immunoblots similar to those shown in **Figure 3.2**. These results, which are shown in **Figure 3.3**, demonstrate for the first time that cofilin is a protein whose in vivo expression is associated with the presence of oligodendrocytes. However, what remains unclear is whether cofilin is an oligodendrocyte-derived protein or a protein generated by a second cell type whose expression is dependent upon and/or regulated by oligodendrocytes. In addition to cofilin, we also detected changes in the expression pattern of the protein stathmin in the X-irradiated optic nerve deprived of oligodendrocytes.

LC-ESI-MS/MS

We identified, by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), additional proteins in the IEF 2-D PAGE gels. Of the approximately ~620 consistent protein spots present on the gels, we chose 19 that showed

Figure 3.3

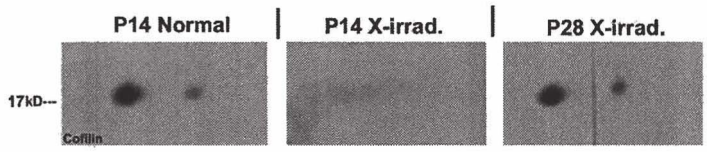


Figure 3.3 The identity of a novel protein associated with oligodendrocytes. Immunoblots of 2-D gels demonstrating the expression pattern of cofilin in the normal and X-irradiated optic nerve at postnatal days (P) 14 and 28. Its expression pattern mirrors that of the known oligodendrocyte proteins (see Fig. 3.2), however, it is not known to be expressed by oligodendrocytes.

at least a 2.00 fold optical density change between those spots expressed in the normal P14 nerve gels and absent and/or down-regulated in the P14 X-irradiated nerve gels **(Fig. 3.4)**. All protein spots followed the characteristic pattern of expression for oligodendrocyte-associated proteins by reappearing in the P28 X-irradiated optic nerve gels. These selected protein spots were excised from the gels of P14 normal rat optic nerves, analyzed by mass spectrometry, and their MS/MS spectra were compared to theoretical spectra in protein databases **(Table 3.1)**. Further immunoblotting and immunohistochemical techniques must still be utilized to verify the identities of the proteins given by our mass spectrometric analysis and database search.

Figure 3.4

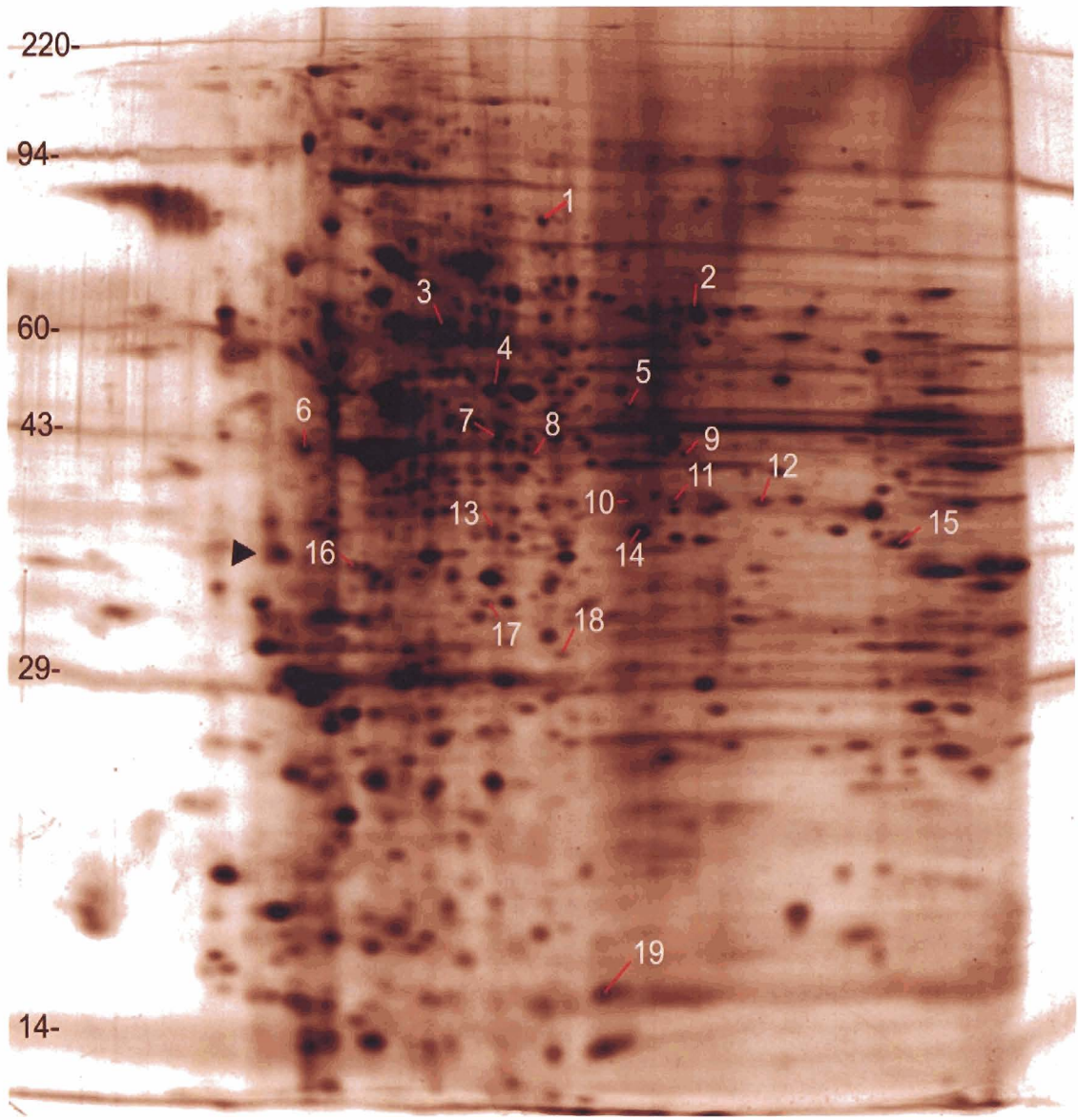


Figure 3.4 Standard IEF-2D-PAGE gel with numbered excised protein spots

Numbers correspond to the excised protein spots analyzed my mass spectrometric techniques (LC-ESI-MS/MS).

Table 3.1

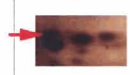
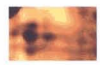
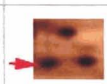
spot ID	Protein Name	Protein Function	~MW (kDa)	~pI	Fold Δ	P14N	P14X	P28X
1	theoretical protein product	-	90	6.2	2.13			
2	5-Aminoimidazole-4-carboxamide ribo-nucleotide formyl-transferase	* intermediate in purine biosynthesis pathway	64	7.2	2.41			
3	dihydropyrimidase-like 2	* neuronal development, outgrowth, connectivity, & regeneration	62	5.9	2.60			
4	dynein, cytoplasmic, light intermediate polypeptide 2	* Intracellular organelle transport * Mitosis	50	6.2	2.02			
5	aldehyde dehydrogenase	* synthesis of all-trans-retinoic acid * metabolic barrier	43	6.8	2.53			
6	laminin-binding protein	* bind neuronally-expressed Laminin 2 * oligodendrocyte cell spreading	37	5.3	2.02			
7	Ndufs2 protein	* subunit of mitochondria complex I involved in OXPHOS ATP production	49	6.1	2.02			
8	protein serine/ threonine kinase	* Ca-signaling pathway involved in controlling neuronal migration	48	6.2	2.30			
9	glutamine synthetase	* glutamine synthesis for brain ammonia detox * myelination control	47	6.7	3.54			
10	zebrin II; aldolase C	* glucose metabolism	39	6.4	2.70			
11	protease serine 1	* proteolytic digestion * neuronal plasticity?	36	6.5	3.54			
12	similar to acetyl CoA transferase-like	* protein acetylation	41	7	2.45			
13	Aspartoacylase	* production of acetyl groups for myelin lipid synthesis	36	6.15	2.22			
14	sirtuin (silent mating type information regulation 2 homolog) 2	* Gene silencing, chromosomal stability, & aging * protein deacetylation	35	6.4	6.83			
15	sirtuin (silent mating type information regulation 2 homolog) 2	* Gene silencing, chromosomal stability, & aging * protein deacetylation	35	7.3	2.80			
16	beta tubulin	* cytoplasmic protein axonal dynamics	34	5.5	2.00			
17	mov34 family	* component of larger signal-some complex involved in phosphorylation of transcrip-tion factors during embryonic development	32	6.1	3.22			
18	macropaine subunit nu	* ATP-dependent non-lysosomal proteinase	29	6.3	2.40			
19	similar to histone (15.4 kD) (his-72)	* chromosomal structure &	14	6.9	2.33			

Table 3.1 This table provides a complete listing of oligodendrocyte-derived and associated proteins identified by LC-ESI-MS/MS along with their functions, molecular weights, and pIs. Cropped images from the 2D gels show the protein spots (red arrows) which follow the characteristic pattern of expression for oligodendrocyte-regulated proteins.

Chapter Four

Discussion

As shown in the *results* section of this thesis, we have elucidated a number of proteins derived from or regulated by oligodendrocytes via an experimental model system developed by our laboratory. In this section, I will discuss the implications of our findings in relation to the functional cooperation between oligodendrocytes and other cells of the developing central nervous system.

Oligodendroctye-Associated Proteins Identified by MALDI-MS

In our initial analysis of the NEPHGE 2D-gels, we identified a number of previously known oligodendrocyte derived proteins (PLP, MOBP, MBP), as well as two other proteins previously unknown to be expressed or regulated by oligodendrocytes. These proteins, identified by MALDI-MS as cofilin and stathmin, were shown to be expressed in the normal P14 nerve, absent in the X-irradiated P14 nerve, and re-expressed in the P28 X-irradiated optic nerve, thus demonstrating that the *in vivo* expression of cofilin and stathmin is associated with and/or dependent upon the presence of oligodendrocytes. We are still uncertain as to whether cofilin and stathmin are oligodendrocyte-derived proteins or proteins expressed by another cell type whose expression is dependent upon and/or regulated by oligodendrocytes. Although experiments are ongoing to ascertain this, it is worth noting that immunohistochemical studies indicate that cofilin is not oligodendrocyte-derived (Lena et al., 1991). Moreover, cofilin has been shown to be present in CNS axons and play a role in the reorganization of the cytoskeleton during

process outgrowth. Specifically, cofilin is an important regulator of actin dynamics, through its ability to depolymerize actin filaments (Gungabissoon & Bamburg, 2003). Stathmin is a known axonal microtubule destabilizer (Moreno & Avila, 1998). Interestingly, we and others have shown that the diametric growth of axons to their final sizes is locally regulated by the oligodendrocytes ensheathment of those fibers (Hernandez et al.,1989; Colello et al., 1994; Sadahiro et al., 2000). Since radial growth of an axon requires the reorganization of the axonal cytoskeleton, contact between an oligodendrocyte and an axon could signal where these proteins should be deposited so as to reorganize the cytoskeleton. Therefore, in the absence of oligodendrocytes, as seen in the P14 X-irradiated nerve, these proteins are not found within the optic nerve axons and radial growth is thus diminished.

Oligodendroctye-Associated Proteins Identified by LC-ESI-MS/MS

Following our initial NEPHGE 2D gel and MALDI-MS analysis, we identified 19 possible oligodendrocyte-regulated or derived proteins out of a possible ~620 spots on our standard IEF-2D gels. The 19 protein spots were analyzed by LC-ESI-MS/MS and a database search of our tryptically digested peptide fragments was run against known proteins. This search resulted in 18 positive matches for a variety of protein identities and 1 theoretical protein product (refer to **Table 3.1**).

Oligodendrocyte-Derived Proteins

A number of proteins identified have previously been classified as oligodendrocyte-derived proteins. These proteins, which showed the characteristic pattern of

oligodendrocyte expression in our 2D gel sets, include **glutamine synthetase, aldehyde dehydrogenase, aspartoacylase, and laminin-binding protein**. *Glutamine synthetase*, an enzyme that plays a critical role in the detoxification of brain ammonia, catalyses the synthesis of glutamine from ammonia and glutamate through the use of ATP (Martinez-Hernandez et al., 1977; Baas et al., 1998). This crucial enzyme is expressed by all macroglial cell types (oligodendrocytes and astrocytes) (Cammer, 1990; D'Amelio et al., 1990; Fressinaud et al., 1991; Baas et al., 1998). It is known that oligodendrocyte development is, in part, controlled by hormone levels. 3,5,3'-Triodo-L-thyronine (T3) hormone induces the differentiation of progenitor cells into developing and myelinating oligodendrocytes (Baas et al., 1994). Studies have shown that *in vitro* and *in vivo* glutamine synthetase expression by oligodendrocytes is modulated and/or regulated by levels of T3 hormone and hydrocortisone (Patel et al., 1983; Frassinaud et al., 1991; Baas et al., 1998). Because glutamine synthetase expression in oligodendrocytes is subject to hormonal regulation, and hormones have a role in the development of myelinating oligodendrocytes; it has been suggested that this enzyme plays a role in the control of myelination (Baas et al., 1998). It is therefore understandable why we see an absence of glutamine synthetase expression in the P14 X-irradiated optic nerve gels.

Aldehyde dehydrogenase is an enzyme that synthesizes all-*trans*-retinoic acid, a transcriptional activator of various cytokines responsible for the physiological response to injury in the nervous system (Raivivich et al., 1999). Dependent upon the aid of NAD+ as a co-substrate, it has been shown in cell culture studies that aldehyde dehydrogenase oxidizes all-*trans*-retinal/retinaldehyde to all-*trans*-retinoic acid (Mey & Hammelmann, 2000), thus playing a crucial role in the response to acute injury in the central nervous

system. Zimatkin and Ostrovskii (1988) have also suggested aldehyde dehydrogenase maintains the role of a metabolic barrier to aldehydes on a systemic level between interstitial fluid and neurons.

Aspartoacylase, another protein displaying the characteristic pattern of oligodendrocyte expression in our 2D gel sets, is an enzyme that has been shown to deacetylate N-acetylaspartate to produce free acetate and aspartate in the brain (Mehta & Namboodiri, 1995; Kirmani et al., 2002a). Aspartoacylase, restricted primarily to myelin producing cells in the CNS (Kirmani et al., 2002b), is a crucial factor in the synthesis of myelin, due to the fact that N-acetylaspartate is the major source of acetyl groups for myelin lipid synthesis (Chakraborty et al., 2001).

Laminin-binding proteins are found in many different cell types. They make up a variety of cell membrane receptor proteins which bind laminin with differing affinities (Smalheiser & Schwartz, 1987). Laminin is a large extracellular protein that affects neuronal behavior, including proliferation, migration, target recognition, neurite outgrowth, and central synaptic differentiation (Yamada, 1983; Smalheiser & Schwartz, 1987; Soussand et al., 2001; Dove, 2003). It has been shown that neuronal expression of laminin 2 induces cell spreading and/or myelination by oligodendrocytes via signaling through β1-integrins and an integrin-linked kinase found to be expressed by oligodendrocytes (Chun et al., 2003).

Oligodendrocyte-Associated Proteins

Through our endeavors, we have also identified a number of proteins expressed by other cells within the CNS whose expression levels appear to be dependent upon and/or

regulated by oligodendrocyte presence. Most of these proteins have been found to be expressed by neurons, the functional unit of the CNS, while others have been shown to have a more diffuse expression pattern among various cell types. Neurons and oligodendrocytes are known to contain vast amounts of mitochondria due to the fact that CNS functioning requires an extraordinary energy capacity. Due to this biophysical characteristic, we have also identified mitochondrial proteins whose expression is shown to be diminished by our X-irradiation model.

Dihydropyrimidinase-like 2 (DHP-like-2) protein, homologous to dihydropyrimidinase-related protein-2, collapsin response mediator protein-2 (CRMP-2), and TOAD-64, is an extracellularly-regulated cytosolic phosphoprotein whose role in neuronal path-finding, migration, and growth-cone collapse is well documented (Hamajima et al., 1996; Arimura ea al., 2000; Castegna et al., 2002; Reibel et al., 2003; Suzuki et al., 2003). Gu and Ihara (2000) have suggested that this group of proteins is also involved in the regulation of microtubule dynamics, particularly *tubulin* (Fukata et al., 2002), a cytoplasmic protein which contributes to axonal growth. DHP-like-2 protein and its homologues are abundantly found in the CNS throughout the stages of early neuronal development (Takemoto et al., 2000). Due to their role in neuronal development, it is clear as to why we see such a dramatic down regulation of protein expression in both DHP-like-2 protein as well as *β-tubulin* in our P14 X-irradiated optic nerve.

Additionally, we found that *cytoplasmic dynein light intermediate chain protein* was down-regulated in our X-irradiation model. Cytoplasmic dynein is a multi-subunit microtubule-associated motor protein involved in retrograde organelle transport, as well

as chromosomal mitotic activity (Hughes et al., 1995; Steffen et al., 1997). Its biochemical and structural similarity to axonemal dyneins makes us question whether our database search overlooked this neuronal protein for one with close homology. Regardless, a down-regulation of dynein expression due to X-irradiation is understandable due to its role in cellular and axonal dynamics.

Oligodendrocytes and other cells of the CNS require enormous amounts of energy to sustain their critical functions. This energy is derived from the vast amounts of mitochondria found within these cells. With the X-irradiation treatment, mitochondria are lost due to the elimination of oligodendrocytes, thus, decreased expression of *NADH-ubiquinone oxidoreductase 49 kDa subunit-2 (Ndufs2),* a subunit of mitochondrial complex I involved in oxidative phosphorylation ATP production (Murray et al., 2003) is reasonable.

A number of *serine-threonine kinases* are expressed throughout the body and have been shown to mediate both stimulatory and inhibitory signals for differentiation and growth of cells. Some serine-threonine kinases are expressed predominantly in the CNS and are involved in a calcium-signaling pathway that mediates neuronal migration (Ryden et al., 1996). It has also been suggested by Ryden et al. (1996) that certain CNS specific serine-threonine kinases are involved in maturation and maintenance of neurons. Thus, with the elimination of oligodendrocytes through X-irradiation treatment, neuronal migration and maturation is interrupted. It would appear that the expression of serine threonine-kinase proteins expressed by neurons within the CNS is dependent upon and/or regulated by oligodendrocytes.

The CNS requires enormous amounts of glucose for proper neurophysiological functioning. Our results show decreased expression of *zebrin II/aldolase C*. Zebrin II is a protein that has previously thought to have been expressed exclusively in cerebellar Purkinje cells (Ahn et al., 1994); however, its homolog, aldolase C, is ubiquitously expressed within CNS neurons. It functions as a glycolytic isozyme that catalyzes the hydrolysis of fructose-1,6-bisphosphate into three carbon moieties in the glycolysis pathway (Lebhertz and Rutter, 1969; Ahn et al., 1994; Walther et al., 1998; Staugaitis et al., 2001). Our data would suggest that the neuronal expression of aldolase C is dependent upon and/or regulated by oligodendrocytes.

Cellular parameters, like energy status for example, are linked to the functioning of chromatin within the nucleus (Denu 2003). The structure, and thus functioning of chromatin is controlled by multiple remodeling enzymes. Our data has shown that the expression one chromatin remodeling enzyme within the CNS, *sirtuin-2 (Sir2)*, is dependent upon oligodendrocyte presence. Nuclear, cytoplasmic, and mitochondrial Sir2 enzymes, with the help of coenzyme NAD+, catalyze a protein deacetylation reaction generating a novel second messenger involved in cell-cycle progression, gene silencing, and chromosomal stability (Landry et al., 2000; Denu, 2003). Transcriptional regulation via chromatin restructured-gene silencing is a method utilized by cells to render regions of a genome transcriptionally inactive (Landry et al., 2000). Although it is clear that X-irradiation treatment successfully eliminates the expression of Sir2 within the developing rat optic nerve, we are uncertain as to whether this implicates a role in which oligodendrocytes may regulate neuronal chromosomal function or whether Sir2 is a protein specific to oligodendrocytes that is lost due to oligodendrocyte elimination.

We identified a change in expression levels of *protease serine 1* due to our X-irradiation model. Protease serine 1 has never been shown to be expressed in the central nervous system; however, its relative CNS serine protease, Neuropsin, plays a significant role in neuronal plasticity and proteolytic digestion of myelin proteins (Terayama et al., 2005). For this reason, it is questionable whether our database search overlooked this CNS expressed protein for one with homology.

A decrease in ubiquitously expressed *macropaine subunit nu* was shown as a result of oligodendrocyte elimination in the optic nerve. This subunit is a part of a larger ATP-dependent, non-lysosomal, multicatalytic proteinase complex (www.hprd.org). At this time it is unknown whether this protein complex is oligodendrocyte-derived or expressed by another cell type whose expression is dependent upon and/or regulated by oligodendrocyte presence.

A number of other proteins were shown to change as a result of elimination of oligodendrocytes from the rat optic nerve. *Mov34*, a component of a larger signalsome complex involved in phosphorylation of transcription factors during development (Bounpheng et al., 2000), was found to decrease expression due to the X-irradiation treatment model. *5-Aminoimidazole-4-carboxyamide ribonucleotide formyltransferase/IMP cyclohydrolase (AICAR)*, a neuro-protective intermediate bi-functional enzyme expressed in neurons involved in *de novo* purine synthesis (Heron et al., 2004), has been shown to promote neuronal survival following cellular stress through the activation of a protein kinase pathway (Culmsee et al., 2001). Our data demonstrates a decrease in expression of AICAR following the elimination of oligodendrocytes. Two proteins with homology to *acetyl-CoA-transferase* and *histone-72* were also shown to be

down-regulated due to X-irradiation treatment. For many of these proteins, it is unclear whether they are expressed specifically by oligodendrocytes or whether their expression by other cells within the CNS is dependent upon the presence of oligodendrocytes.

In conclusion, we have utilized a unique *in vivo* model system to establish, for the first time, a list of proteins whose expression pattern is associated with oligodendrocytes. Future studies into the molecular and biochemical properties of the identified proteins, correlated with their immunocytochemical localization in ultra-structurally distinct myelin and axonal membranes can provide further clues to their possible roles in the development of the CNS. This is extremely important due to the fact that oligodendrocyte-derived and regulated protein expression is crucial to the health and functioning of the developing and mature CNS. Its importance can be measured by the profound sensory and motor deficits incurred as a result of diseases that target and destroy oligodendrocytes and myelination. The consequences of oligodendrocyte dysfunction are devastating to myelinogenesis and to the normal transferring and processing information by the mature CNS. It is our hope that researchers will find this updated protein list useful in directing the scope of future endeavors aimed at diminishing the effects of oligodendrocyte dysfunction.

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Vita

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